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Applying population genetics to define the units for conservation management in the European Tree Frog, *Hyla arborea*

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***Abstract.*—Population genetic analyses are a powerful tool for obtaining information about cryptic genetic lineages, population structure, and the distribution of intra- and interpopulation genetic diversity across the landscape. This knowledge is crucial for establishing units for the conservation management of endangered species. Species with limited dispersal capacities, such as amphibians, are particularly affected by habitat fragmentation and reductions in gene flow among isolated populations. The European Tree Frog, *Hyla arborea*, has suffered from dramatic population declines in the last decades and is categorized as Vulnerable to Critically Endangered in its north-western distribution range. In Lower Saxony (Germany), the current distribution of the tree frog is fragmented. In this study, we aimed to assess the population structure, genetic diversity, gene flow, and migration rates in order to define the units for conservation management. Across a distribution area of 250 km², frogs were sampled at 14 localities and genotyped at seven microsatellite loci, and the *mt*DNA cytochrome *b* gene was sequenced for a subsample. Whereas microsatellite pairwise D_{est} and F_{ST} values showed genetic differentiation among nearly all sampled populations, Bayesian analyses assigned the 14 localities to two distinct genetic clusters including seven subclusters. Together with a slight correlation between geographic and genetic distance, the population structure indicates ongoing fragmentation. The cytochrome *b* haplotype distribution does not indicate divergence into *mt*lineages, but highlights the former connection of populations along the river Elbe. The results of this study suggest that the intense anthropogenic pressures in this area over the last decades have had negative genetic consequences for this species. The fragmented population structure calls for reconnection of the isolated occurrences by the implementation of conservation measures.**

Keywords. Amphibian, Bayesian assignment, conservation genetics, genetic diversity, population fragmentation, population structure

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Introduction

Genetic diversity and connectivity mediated by migrating individuals between populations are critical for the maintenance of many threatened species and can be evaluated by population and landscape genetic analyses (Shaffer et al. 2015). Loss of connectivity disrupts gene flow between formerly connected habitats and leads to the isolation of populations. Isolation in turn imposes a more rapid erosion of genetic diversity, exacerbating the effects of genetic drift and inbreeding on local gene pools (Andersen et al. 2004; Crnokrak and Roff 1999; Hedrick and Kalinowski 2000; Luquet et al. 2011).

When reconnection of the habitats of endangered species is necessary, it is essential to determine the genetic structures and migration patterns for effective conservation management. This information can be used to delineate conservation units (e.g., Palsbøll et al. 2007), even though the concepts that are applied to define them are somewhat uneven among studies and taxa (Shaffer et al. 2015). While Evolutionary Significant Units (ESUs) are used to delineate entities which possess a long (evolutionary) history (Crandall et al. 2000; Moritz 1994), management efforts are often restricted to a more recent and regional space. In such cases, population boundaries need to be identified among which gene flow is limited. To achieve this, both mitochondrial

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and nuclear markers are informative. Mitochondrial DNA has been widely used to analyze the phylogenetic relationships of amphibian populations (Dufresnes et al. 2013; Stöck et al. 2012), while nuclear markers like microsatellites are well suited for detecting fine-scale structuring of populations and recent loss of genetic variation (Selkoe and Toonen 2006). Population genetic approaches such as Bayesian assignment tests use population allele frequencies to group individuals into genetic clusters. Together with information on genetic divergence between genetic clusters, this approach can be used to denote conservation units (Olsen et al. 2014; Rowe and Beebee 2007).

In Europe, habitat loss, fragmentation, and degradation – mostly due to anthropogenic pressure (Cushman 2006; Pimm and Raven 2000) – are the most significant threats to endangered wildlife populations (Fahrig and Merriam 1994; Sih et al. 2000; Stuart et al. 2004). Amphibian populations are especially vulnerable to fragmentation and loss of genetic variation due to their low dispersal capabilities (as reviewed in Smith and Green 2006). For safeguarding vulnerable species of this most endangered vertebrate group (Stuart et al. 2004), it is necessary to counteract genetic depletion by maintaining the exchange of individuals among populations.

The European Tree Frog has shown long-term decline in much of its Western European distribution, mainly caused by habitat fragmentation (Andersen et al. 2004; Dubey et al. 2009; Krug and Pröhl 2013). The highest genetic diversity of this species has accumulated in South-eastern Europe, where it survived in refugia during glaciations. After late-Pleistocene diversification on the Balkan Peninsula, one of several major genetic groups recolonized North and Western Europe. Postglacial expansions resulted in decreasing genetic diversity across the range and therefore increased the vulnerability of populations towards North-Western Europe (Dufresnes et al. 2013; Stöck et al. 2012). Indeed, the tree frog is not categorized as threatened in South-Eastern Europe, while it is reported to have declined and is now classified as Vulnerable to Critically Endangered in different areas in the north-west (see review in Dufresnes et al. 2013, Table S1). In Lower Saxony in Germany, where the current distribution is patchy with some main occurrences in the lowlands (Fig. 1), the conservation status of the tree frog is Endangered (see the Red List at http://www.amphibienschutz.de). Although the species was widespread in the past, severe declines have been observed mainly in the second half of the last century (Manzke and Podloucky 1995). In some places, measures for conservation management have been successful (Brandt and Lüers 2017; Buschmann et al. 2006; Richter and Mügge 2012).

For supporting further conservation activities, analyses of the genetic structure are required for assessing the genetic clusters as a way to delineate the units for conservation management (Rowe and Beebee 2007). So far, several studies have measured the genetic structure and diversity in more or less fragmented metapopulation systems (Andersen et al. 2004; Angelone and Holderegger 2009; Arens et al. 2006; Dubey et al. 2009; Edenhamn et al. 2000; Krug and Pröhl 2013). The aim of this study was to perform a conservation genetic survey of the European Tree Frog across its distribution in Lower Saxony and adjacent areas. The specific intention was to assess significant genetic differentiation in order to define those conservation units among which dispersal is restricted. The obtained information was then used to identify population management goals and to provide specific recommendations about conservation priorities to ensure the long-term survival of the tree frog in this region.

In this study, we tested hypotheses regarding population genetic structure, differentiation, and diversity in the Endangered European Tree Frog by analyzing mitochondrial sequence and nuclear microsatellite data with a series of statistical techniques. First, we tested for the existence of diverged genetic lineages. We further predicted that past population expansion and recent habitat fragmentation 1) reduced the migration among localities; 2) reduced genetic diversity as well as genetic population size within localities; and 3) resulted in significant genetic structure among the remaining tree frog localities. Therefore, 4) we expected a small to moderate effect of geographic distance on genetic differentiation as a result of the ongoing population disconnection. This work reveals ongoing population fragmentation with moderate genetic diversity for the populations of the European Tree Frog in Lower Saxony.

Materials and Methods

Sample Collection and Preparation

Fourteen sites were sampled across the tree frog distribution in Lower Saxony and adjacent distributions in North Rhine Westphalia and Saxony-Anhalt, all in Germany. We chose one sample site within each main occurrence of the tree frog in this region (Fig. 1). In the occurrence near Hannover, however, we sampled four sites: two in the west of Hannover (KZ, KO, see Table 1 for site definitions) and two in the east of Hannover (KH, BH) for a comparison of smaller scaled spatial distances. In total, 237 individuals were sampled with 5–22 individuals per sample site (Table 1). Genetic material was collected from the tips of tadpole tails and buccal swabs of adult frogs. The adults were collected from the choruses during the breeding seasons in spring 2007 and 2008. Tadpoles were sampled in summer 2007 at three localities. In this year, the climatic conditions for breeding where unfavorable and adult catch rates were low at these sites. To avoid bias in the results from tadpole samples representing offspring from only one breeding pair, tadpoles were sampled in different breeding ponds.

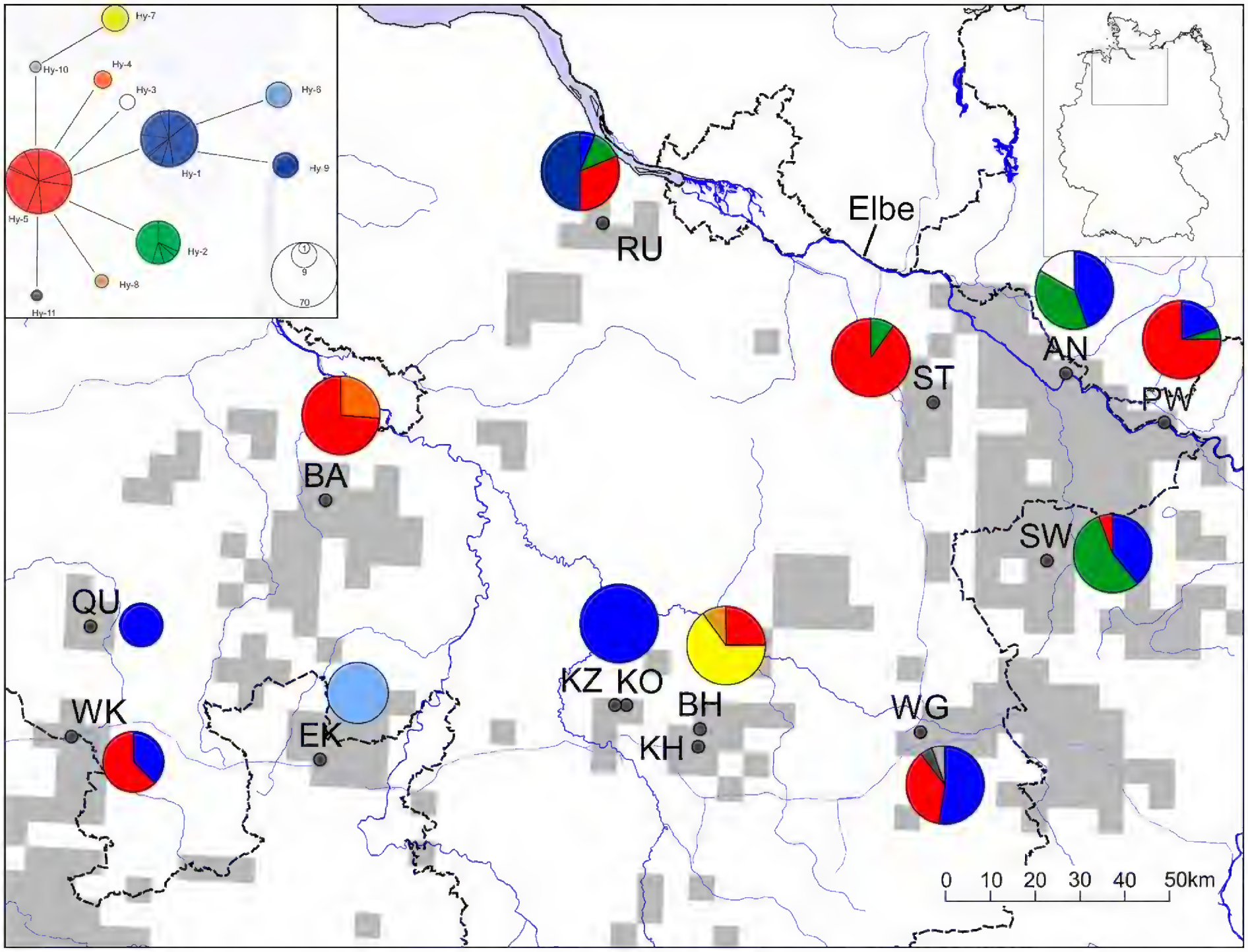

Fig. 1. Current distribution of the European Tree Frog, and the distribution of cyt *b* haplotypes in Lower Saxony and adjacent areas on the basis of TK25-quadrants (grey squares) during 1994–2010 in Lower Saxony (NLWKN 2011), 1993–2006 in North Rhine Westphalia (LANUV 2011), and 1990–2000 in Saxony Anhalt (Meyer et al. 2004). Dashed lines denote state borders, dots denote sample sites. **Inset in upper left corner:** Haplotype network of 11 distinct haplotypes of cyt *b* of *H. arborea* (901 bp) in Lower Saxony and adjacent areas. Each haplotype is represented by one circle and color. The size of a circle corresponds to the haplotype frequency. Lines between haplotypes denote mutational steps between sequences.

The genetic diversity for these localities was similar to other locations, suggesting that relatedness among samples did not bias the results (Table 1). DNA from the tail clips was fixed in 99% ethanol and extracted using a proteinase K digestion followed by a Phenol-Chloroform protocol (Sambrook et al. 1989), and then stored at -20 °C. DNA was extracted from the buccal swabs with an Invisorb Spin Swab Kit (Invitek) following the manufacturer's protocol, and stored at -20 °C. Another study confirmed that buccal swabbing is a very efficient method for obtaining DNA of adequate quality for microsatellite amplification (Broquet et al. 2007).

A total of seven polymorphic microsatellite loci (WHA1-9, WHA1-20, WHA1-25, WHA1-67, WHA1-103, WHA1-104, and WHA1-140) previously isolated by Arens et al. (2000) were amplified following the author's protocol, except that the annealing temperature for WHA1-20 was changed to 64.6 °C. The PCR products were genotyped using the capillary sequencer MegaBace 1000 (Amersham Bioscience). Allele scoring was performed using the software Genetic Profiler v. 2.2. The genotyping results can be found in the file that accompanies this article (Supplementary file 1).

Because earlier analyses (Stöck et al. 2012) included only four samples of mt DNA from Germany, we also sequenced cytochrome *b* (cyt *b*) fragments of 901 bp for 5–20 individuals from each sample site, excluding KO and BH from the Hannover population. The cyt *b* fragment was amplified via PCR using the primers MVZ 15-L (5′-GAACTAAT GGCCCA CACWWTACGNAA -3′) and cyt *b* AR-H (TAWAAGGGTCTTCTACTGGTTG) from Moritz et al. (1992) and Goebel et al. (1999). The PCR reaction (25 µl) consisted of 20–100 ng DNA, 1 µl of each primer (10 µM), 0.8 µl dNTPs (10 mM, 5PRIME), 2.5 µl 10x advanced Buffer (5PRIME), 1.25 U Taq DNA Polymerase (5PRIME), and 17.45 µl H_2O. The PCR conditions were as follows: an initial denaturation at 94 °C for 3 min; 35 cycles at 94 °C for 45 s, annealing temperature of 50 °C for 45 s, and extension at 65 °C for 1 min. The PCR products were sent to the Macrogen Company (Seoul, South Korea) for purification and sequencing with an ABI3730XL genetic analyzer (Applied Biosystems).

Table 1. Overview of data from the various sample sites. [a]: Samples from adult frogs, [t]: samples from tadpoles, H_o: observed heterozygosity, H_e: expected heterozygosity, SD: standard deviation, F_{IS}: inbreeding coefficient, with bold values for significant differences after 1,000 permutations, R: mean allelic richness over all loci, *h*: haplotype diversity, π: nucleotide diversity, *N*: number of sampled individuals, N_A: mean number of alleles over all loci.

ID	Sample site	mean $H_o \pm$ SD	mean $H_e \pm$ SD	F_{IS}	N_A	R	*h*	π [%]	*N*
QU	Quakenbrück [a]	0.786 ± 0.248	0.741 ± 0.143	-0.070	4.00	–	0.00	0.00	5
WK	Westerkappeln [a]	0.661 ± 0.187	0.579 ± 0.158	**-0.154**	3.43	–	0.54	0.06	8
EK	Espelkamp [a]	0.796 ± 0.169	0.754 ± 0.087	-0.058	5.29	5.18	0.00	0.00	12
KZ	Kananohe Zentrum [a]	0.667 ± 0.169	0.666 ± 0.134	-0.001	5.14	4.65	0.00	0.00	20
KO	Kananohe Ost [a]	0.701 ± 0.208	0.684 ± 0.089	-0.027	4.57	4.53	–	–	11
KH	Kolshorn [a]	0.754 ± 0.131	0.713 ± 0.094	-0.059	6.29	5.37	0.53	0.13	20
BH	Beinhorn [a]	0.693 ± 0.089	0.693 ± 0.093	-0.001	5.57	4.84	–	–	20
BA	Bassum [t]	0.771 ± 0.099	0.748 ± 0.092	-0.032	5.43	4.98	0.41	0.05	20
RU	Ruschwedel [a]	0.731 ± 0.064	0.721 ± 0.050	-0.015	5.00	4.50	0.68	0.14	18
WG	Wolfsburg-Gifhorn [a]	0.790 ± 0.158	0.799 ± 0.080	-0.011	7.71	6.65	0.61	0.08	20
ST	Strothe [a/t]	0.735 ± 0.153	0.708 ± 0.126	-0.039	6.29	5.34	0.19	0.02	21
AN	Amt Neuhaus [a]	0.708 ± 0.111	0.750 ± 0.090	0.057	6.43	5.50	0.66	0.15	22
SW	Salzwedel [t]	0.600 ± 0.227	0.687 ± 0.181	**0.130**	6.00	5.01	0.57	0.11	20
PW	Pevestorfer Wiesen [a]	0.793 ± 0.110	0.764 ± 0.091	-0.039	6.43	5.55	0.42	0.05	20

Statistical Analysis

Analysis of mtDNA

Both directions of the cyt *b* sequences were assembled using the computer software SeqMan™ II (DNASTAR, Inc., Konstanz, Germany). Multiple sequence alignments were performed in MEGA 4 (Tamura et al. 2007) using the Muscle algorithm (Edgar 2004), and all variable sites were confirmed by visual inspection of the chromatograms. The EMBL-EBI sequence analytical tool (Madeira et al. 2022) was used to convert the sequences to the corresponding amino acid sequences in order to assure that nuclear copies were not sequenced. The program MEGA was applied to calculate *p*-distances between sample sites (Tamura et al. 2004). Haplotype diversity (*h*) and nucleotide diversity (π) (Nei 1987) were determined with Arlequin ver. 3.11 (Excoffier et al. 2005). A haplotype network of the cyt *b* data set was constructed via the statistical parsimony analysis of the program TCS 1.21 (Clement et al. 2000) using the default settings.

Analysis of Microsatellites

Microsatellite data were checked for null alleles, stuttering, and allelic dropout using Micro-Checker (Van Oosterhout et al. 2004). The program Fstat v. 2.9.3 (Goudet 1995) was used to test for genotypic disequilibrium of all pairs of loci in each sample and to calculate average allelic richness per population. For the calculation of average allelic richness, sample sites with less than ten individuals (QU and WK) were excluded.

For each sample site and locus, the observed and expected heterozygosity (Nei 1987) and deviation from Hardy-Weinberg equilibrium (HWE) (Guo and Thompson 1992) were determined with Arlequin ver. 3.11 (Excoffier et al. 2005). Genepop ver. 4.1 (Rousset 2008) was used to test for a global deviation from HWE in each sample site. The inbreeding coefficient F_{IS} per sample site (Weir and Cockerham 1984) was calculated using Genetix ver. 4.05 (Belkhir et al. 2004) and the significance was tested with a permutations test (1,000 permutations).

Genetic differentiation between the sample sites was calculated as global F_{ST} and pairwise F_{ST} values (Weir and Cockerham 1984) in Arlequin (Excoffier et al. 2005). In addition, pairwise D_{est} (Jost 2008), a substitute measure of genetic differentiation, was calculated using the R package DEMEtics (Gerlach et al. 2010). Significance was calculated by 10,000 bootstraps.

The data were also tested for Isolation By Distance in sampled populations (IBD; Storfer et al. 2010; Wright 1943). IBD occurs when gene flow occurs but declines with increasing distances between pairs of populations, and is typical for the genetic population structure of many animal species (Hitchings and Beebee 1997; Spear et al. 2005; Vergara et al. 2015). To test for IBD, a Mantel test for correlation between pairwise genetic distances (F_{ST} and D_{est}) and pairwise geographic distances was conducted, implemented in IBDWS 3.23 (Jensen et al. 2005). As proposed by Rousset (1997) for populations in two-dimensional habitats, geographical distance was log-transformed and genetic distance was expressed as $F_{ST}/(1 - F_{ST})$, and $D_{est}/(1 - D_{est})$. Significance for $r \geq 0$ was assessed via 10,000 bootstraps. The linear geographic distances among sample sites were calculated in ArcView GIS 3.3 using the Distance Matrix extension (Jenness 2005).

Even though the Mantel test is widely used in landscape genetic studies, an evaluation of different methods revealed that Mantel tests exhibit high type-1 error rates (Balkenhol et al. 2009). Those authors recommended applying a combination of statistical methods to avoid inaccurate conclusions derived from only one method. Therefore, two additional hierarchical Bayesian methods, GESTE (Foll and Gaggiotti 2006) and BIMr (Faubet and Gaggiotti 2008), were applied here for evaluating the effect of distance by means of generalized linear models. Both BIMr and GESTE perform well for moderate samples sizes and limited numbers of loci, as in our study (Balkenhol et al. 2009).

GESTE estimates the genetic distance (F_{ST} values) for each local population pair from multilocus genotypes and correlates them to environmental factors. Posterior probabilities associated with each factor allow the identification of factors with the highest effect on genetic structure. The regression coefficient estimate (Alpha) indicates whether a factor reduces or enhances genetic differentiation. The estimation of model parameters is performed by using a combination of Markov Chain Monte Carlo (MCMC) and Reversible-Jump MCMC (RJMCMC) (Green 1995). As environmental factors, we included latitude (G1) and longitude (G2; geographic coordinates in GK3 format) as approximations of the effect of distance among population pairs.

The software BIMr 1.1 estimates contemporary gene flow and assesses the influence of genetic distance on gene flow. This program quantifies the gametic disequilibrium from multilocus genotypes (here, microsatellite alleles) generated by the progeny of recent migrants to calculate the proportion of the population that immigrated during the last generation (Faubet and Gaggiotti 2008). Five replicates (= runs) were run with a total of 1,020,000 iterations (burn-in: 1,000,000, sample size: 20,000) and a thinning interval of 50 iterations. For each replicate, first 20 short pilot MCMC runs of 1,000 iterations were conducted, and the run with the lowest Bayesian deviance (D_{assign}) and the highest posterior probability was selected to extract the parameter estimates (Faubet et al. 2007; Faubet and Gaggiotti 2008). Two models were calculated: model 0 did not include environmental factors and model 1 included factor G1 which is the geographic distance between pairs of populations. As an alternative, the BayesAss software (Rannala 2007; http://www.rannala.org/software/) was also used to infer contemporary migrations rates. The software was run with 10,000,000 iterations (i), a burn-in (b) of 1,000,000 repetitions, and the interval between samples (n) was set to 1,000. The default values were used for all other parameters at first. Then, we adjusted the mixing parameters for migration rate (m), allele frequencies (a), and inbreeding coefficients (f) to maintain their acceptance rates between 20% and 60% as recommended in the Manual.

To infer genetic clusters, individual assignments to populations were conducted by means of a combination of non-spatial and spatial Bayesian algorithms with Structure version 2.3.3 (Pritchard et al. 2000) and TESS version 2.3 (Chen et al. 2007; François et al. 2006). Simulation data suggested the combination of TESS and Structure as a reliable approach for deducing the spatial population structure (Chen et al. 2007), outperforming other Bayesian clustering programs. All Structure runs used 500,000 iterations after a burn-in period of 100,000. An admixture ancestry model and correlated allele frequencies were used between populations. Structure was run both without and with information about the sampling location (prior population information) and the results were compared as recommended by Pritchard et al. (2000; see also Dufresnes et al. 2013; Olsen et al. 2014). Hierarchical analyses were performed by repeating the Structure runs with each of the major clusters. Twenty runs were conducted for each K. The range of possible Ks tested spanned from 1 to 14, according to the number of sampled breeding sites. The average log likelihood Pr(X|K) (given by the estimated Ln Prob of data = Ln P(D) in the software result output, see Table 4) was calculated for each K across all runs. Since detecting the true number of K is not always straightforward, we included the ΔK statistics proposed by Evanno et al. (2005), using Structure Harvester v.0.6.8 (Earl and von Holdt 2012).

TESS uses a Bayesian method to detect population structure, but it considers the spatial information (geographical coordinates) of the individuals. After assessing the preliminary runs as recommended in the software manual, the maximum number of allowed genetic clusters (K_{max}) was varied from 2 to 10. One hundred independent runs for each K_{max} were conducted under the admixture model, with 50,000 sweeps and a burn-in period of 10,000 sweeps for each run.

Finally, the NeEstimator v2 software was used to estimate the contemporary effective population sizes at all sample sites (Do et al. 2014). Three single sample estimators were implemented: the linkage disequilibrium method, the heterozygote-excess method, and the molecular coancestry method. The lowest allele frequencies (P_{Crit}) were set to 0.05, 0.02, 0.01, and 0+.

Results

Genetic Diversity

Genetic diversity was estimated for each population based on the microsatellite alleles and the cyt *b* haplotypes (Table 1). While genetic diversity indices based on microsatellites (H_o, H_e, N_A, and R) are moderate to high across the range, they are always highest in WG (except for H_o); while the indices based on cyt *b* (h, π) tend to increase from west (WK/QU) to east (PW) (Table 1; Fig. 1).

Table 2. Pairwise D_{est} values (lower matrix) and pairwise F_{ST} values (upper matrix) between sample sites; ns = not significant. See Table 1 for sample site acronym definitions.

	QU	WK	EK	KZ	KO	KH	BH	BA	RU	WG	ST	AN	SW	PW
QU	0	0.120	0.066	0.111	0.113	0.110	0.103	0.039	0.117	0.043	0.086	0.086	0.084	0.052
WK	0.208	0	0.114	0.064	0.107	0.117	0.154	0.097	0.191	0.107	0.083	0.119	0.162	0.147
EK	0.195	0.286	0	0.041	0.031^{ns}	0.069	0.076	0.060	0.090	0.061	0.095	0.065	0.097	0.071
KZ	0.284	0.145	0.102	0	0.002^{ns}	0.090	0.099	0.066	0.139	0.081	0.092	0.081	0.095	0.105
KO	0.324	0.243	0.097	0.001^{ns}	0	0.092	0.104	0.059	0.130	0.081	0.092	0.074	0.091	0.106
KH	0.412	0.301	0.222	0.279	0.290	0	0.025	0.053	0.062	0.064	0.056	0.074	0.110	0.094
BH	0.387	0.372	0.230	0.284	0.311	0.059	0	0.074	0.092	0.082	0.076	0.102	0.118	0.100
BA	0.189	0.230	0.220	0.199	0.189	0.172	0.225	0	0.074	0.037	0.072	0.070	0.083	0.066
RU	0.424	0.449	0.285	0.382	0.386	0.173	0.252	0.235	0	0.068	0.079	0.072	0.110	0.073
WG	0.139	0.309	0.296	0.230	0.306	0.237	0.283	0.142	0.252	0	0.056	0.071	0.086	0.061
ST	0.299	0.208	0.329	0.281	0.308	0.175	0.238	0.239	0.222	0.201	0	0.057	0.110	0.070
AN	0.265	0.315	0.230	0.248	0.263	0.245	0.316	0.242	0.238	0.290	0.211	0	0.097	0.065
SW	0.238	0.391	0.289	0.233	0.228	0.368	0.367	0.272	0.347	0.287	0.369	0.343	0	0.078
PW	0.213	0.365	0.278	0.278	0.322	0.350	0.337	0.253	0.267	0.249	0.226	0.241	0.253	0

Mitochondrial Sequence Analysis

The analysis revealed 11 haplotypes of the cytochrome *b* fragment which differed by ten variable sites and nine parsimony informative sites (Fig. 1). There was no evidence for any diverged haplotype groups that would correspond to different genetic lineages. Most haplotypes were closely related but unique to one sample site, except for haplotypes Hy-1, Hy-2, and Hy-5. While Hy-1 (blue) and Hy-5 (red) showed a broad distribution over almost the complete sampling area, Haplotype Hy-2 (green) was restricted to five sample sites in the northeast (Fig. 1). Eight haplotypes were found at only a single locality: Hy-3 (white) in AN, Hy-4 (orange) in BA, Hy-6 (light blue) in EK, Hy-7 (yellow) and HY-8 (brown) in KH, Hy-9 (dark blue) in RU, and Hy-10 (grey) and Hy-11 (dark grey) in WG. In WG and RU, four different haplotypes were detected, while in QU, EK, and KZ (all in the west of Hannover) only one haplotype was found. The *p*-distances among localities were low, varying between 0 and 0.4 % (Supplementary Table S1). The GenBank accession numbers can be found in Supplementary Table S3.

Microsatellite Analysis

The seven microsatellite markers examined were polymorphic with seven to 16 alleles per locus. The analysis with Micro-Checker uncovered signs of null alleles for locus WHA1-67 in sample site KO and for locus WHA1-140 in sample site SW. As null alleles for these two loci were found at only a single sample site, we did not adjust for null alleles. Furthermore, this analysis revealed no evidence for large allele dropout or scoring errors due to stuttering.

Deviation from Hardy-Weinberg-Equilibrium (HWE) was found for WHA1-104, with a significant excess of heterozygotes in sample site KH. The global test for HWE over all loci in each population resulted in no significant deviation from HWE. Significance values for the inbreeding coefficient F_{IS} were obtained for the sample sites SW (F_{IS} = 0.130) and WK (F_{IS} = -0.154, Table 1). No linkage (genetic) disequilibrium was found between any pair of loci.

The global F_{ST} value across all localities was 0.083 and highly significant ($P > 0.0001$). Genetic differentiation calculated as pairwise D_{est} and pairwise F_{ST} values were significant in all cases except between the two sample sites in the West of Hannover (KZ and KO), as well as EK and KO regarding the F_{ST} values (Table 2). The Mantel test for IBD showed a significant but low correlation between the genetic and geographic distances (Fig. 2; D_{est}: $r = 0.28$, $P = 0.0117$; F_{ST}: $r = 0.29$, $P = 0.0145$, see also Supplementary Table S2), indicating that genetic differentiation is only partially explained by geographic distances among the sites.

GESTE calculated five different models (Table 3). The probability of a model was not improved by including either latitude (G1) or longitude (G2) without interaction. The model with the highest posterior probability was model 4, which included the constant, latitude, and longitude as well as their interaction. The Alpha values were low for the effects of both factors, while the Alpha value of the interaction indicates a significant effect on genetic differentiation.

All five replicates of the BIMr analysis showed a D_{assign} of 0.0. The highest posterior probability for the null model was 0.79 (run 1), and the lowest was 0.55 (run 4). The posterior probabilities for model 1 (including G1) were lower than the posterior probabilities of the null model (Table 4); i.e., the geographic distance did not seem to affect recent gene flow or migration among sample sites. Mean migration rates were extremely low and varied from $2.88e^{-12}$ to $1.11e^{-9}$; while the highest mean migration rate was observed among PW and WG, and the lowest was among AN and QU. Also, the

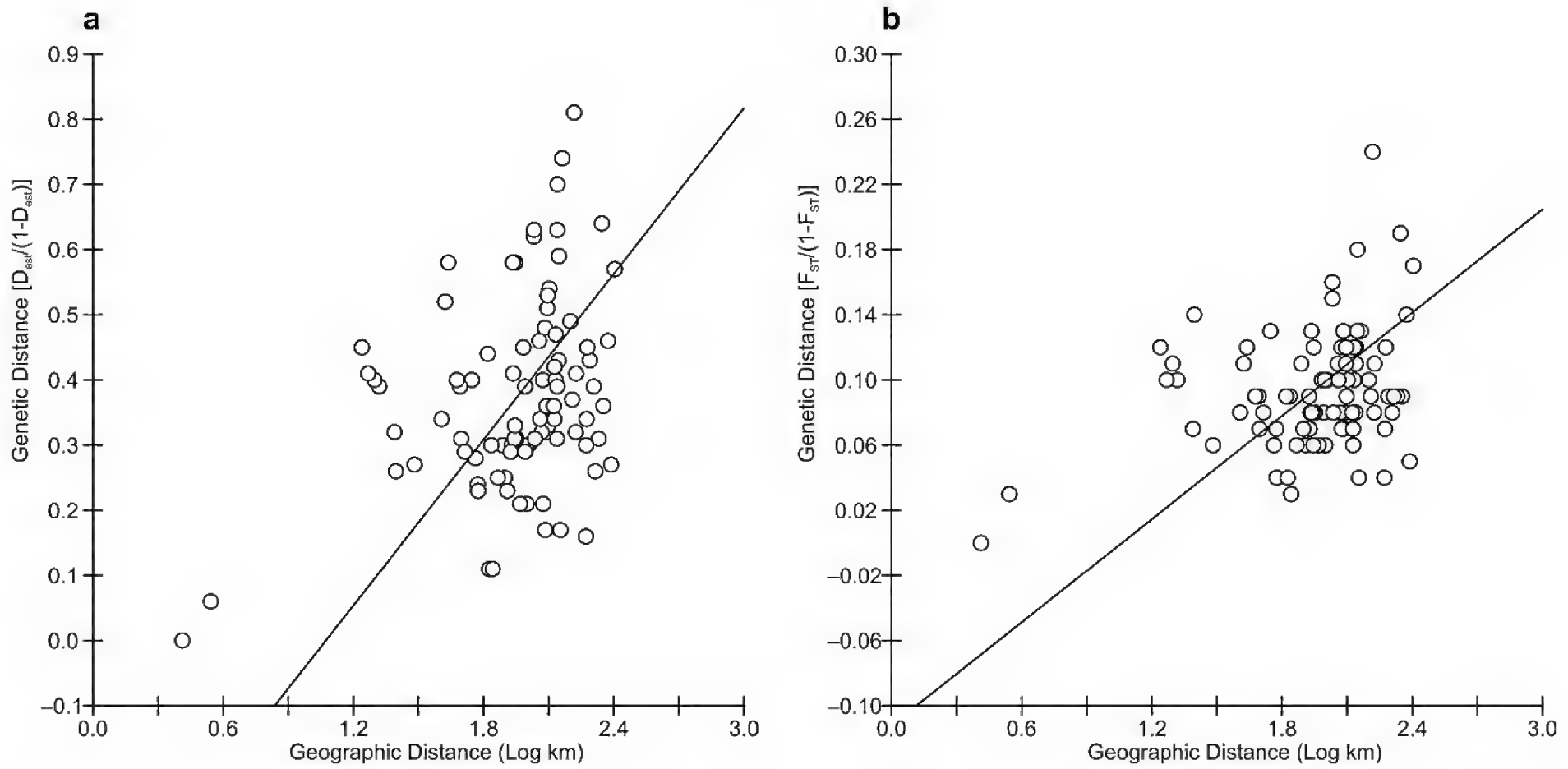

Fig. 2. Isolation By Distance plots. (a) $D_{est}/(1 - D_{est})$ versus log geographic distance; and (b) $F_{ST}/(1 - F_{ST})$ versus log geographic distance. The lines are the RMA (Reduced Major Axis) regressions.

migration rates between two close population pairs in the surroundings of Hannover were very low. For example, the mean migration rates between KH to BH and vice versa were only $1.66e^{-10}$ and $2.34e^{-10}$, and those between KZ and KO were $1.92e^{-10}$ and $2.24e^{-10}$ even though the distances between these pairs were only 3.94 km and 2.6 km, respectively. The analysis with BayesAss provided very similar results for the runs with default and adjusted parameters. The migration rates were higher than those calculated with BIMr, most of which fluctuated around 0.01. Interestingly, the migration rates between EK and KZ, KO and KZ, as well as KH and BH were considerably higher (0.14, 0.16, and 0.20, respectively) and more consistent with the genetic population structure (Fig. 3) as well as the above-mentioned geographic distances.

Bayesian assignments conducted by Structure suggested two major groups (K = 2), separating the western/central populations (WK-BA-EK-KZ-KO-BH-KH) and the northern/eastern populations (RU-WG-ST-AN-PW-SW, Figs. 3A and 4, see also Supplementary Fig. S1A). The population QU appeared to be admixed, but was unambiguously assigned to the western group by TESS (see below). Within each cluster, a fine substructuring could be detected, and the two approaches (with/without prior population information) provided slightly different clustering solutions. In those runs where the sampling location was used as prior information, the western/central cluster was split along Hannover with some admixed populations in the western part of the region (K = 3, Fig. 3B). The Structure runs without prior population information supported the existence of two genetic subclusters within this region (Supplementary Fig. S1B). In the northern/eastern cluster, both approaches provided similar results (Fig. 3C and Supplementary Fig. S1C). RU was differentiated from WG and ST, which in turn differed from the populations lying in the eastern part of the study area in Saxony Anhalt. More detailed results of log likelihood Pr(X|K) values and ΔK statistics are provided in the Supplementary figures (Figs. S2 and S3, respectively).

The TESS analysis supported the two major genetic groups separated into a western/central cluster and a northern/eastern cluster (Fig. 5). Increasing the number of K_{max} resulted in only a slight decrease in the Deviance

Table 3. Posterior probabilities for five possible models calculated with GESTE explaining the genetic differentiation of European Tree Frogs as a function of the environmental factors latitude (G1) and longitude (G2). Constant is the intercept of the regression model. The regression coefficients (Alpha) for different environmental factors used in the models are given in the right side of the table.

Model	Factors included	Posterior probability	Factors	Regression coefficients	
Model 0	Constant	0.19	Constant	Alpha 0	-3.69
Model 1	Constant, G1	0.19	G1	Alpha 1	0.06
Model 2	Constant, G2	0.18	G2	Alpha 2	-0.07
Model 3	Constant, G1, G2	0.17	G1*G2	Alpha 3	-2.14
Model 4	Constant, G1, G2, G1*G2	0.26			

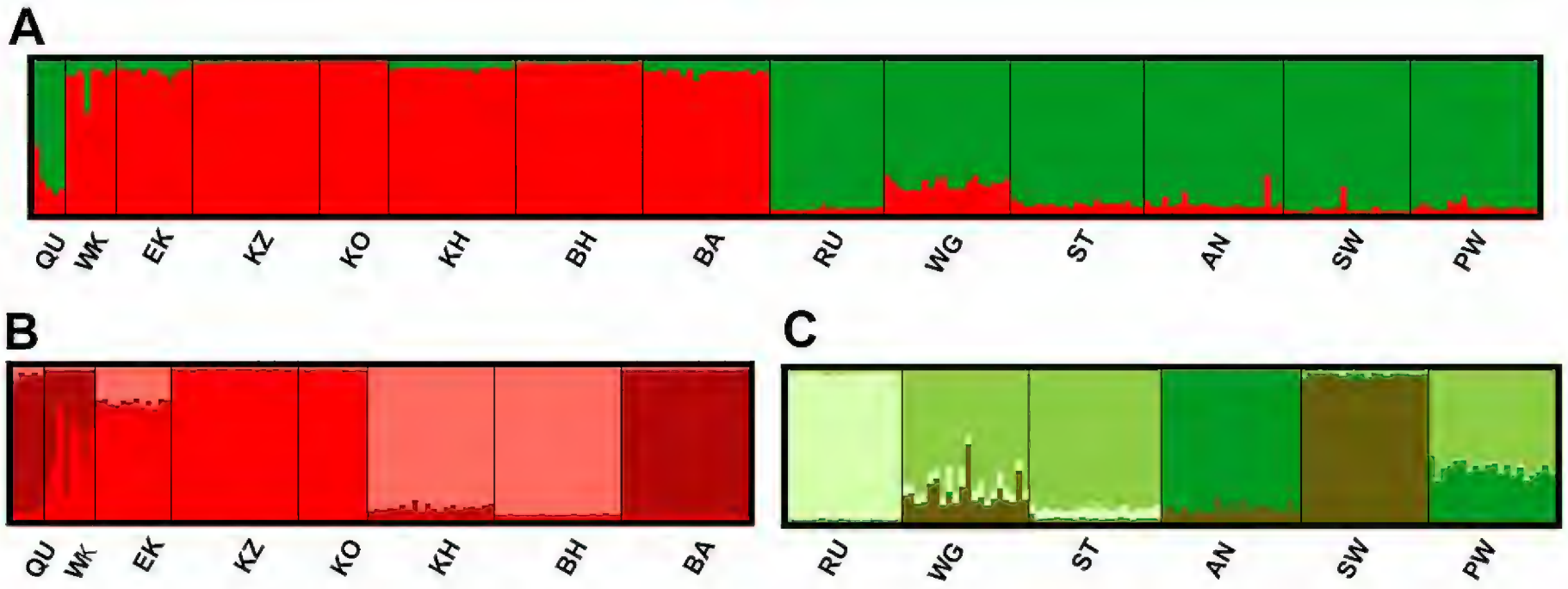

Fig. 3. Estimation of the number of *Hyla arborea* populations using the program STRUCTURE ver 2.3.1 (Pritchard et al. 2000) for the admixture model with prior population information; QU, WK, EK, etc. = sample sites, separated by fine black lines. Each individual is represented by a single vertical line broken into colored segments, with lengths proportional to the corresponding clusters. (A) Plot for $K = 2$ in the analysis of the entire data set, (B) plot for $K = 3$, and (C) $K = 4$ for hierarchical analysis on each of the two main clusters.

Information Criterion (DIC) of the models while the resulting population structure was not consistent among runs for each K_{max} or in comparison with the STRUCTURE results (data not shown). STRUCTURE as well as TESS revealed that frogs from WG in the eastern group (Fig. 3A) are of admixed origin from both genetic groups as the assignment results from the two were similar.

Effective Population Sizes

Mean effective population sizes (N_e) varied among the sample sites and statistical methods applied. The results also differed between $P_{Crit} = 0.05$ and the other P_{Crit} but were the same for $P_{Crit} = 0.02$, 0.01, and 0+ (Table 5). The large confidence intervals indicate that the results might not be very reliable. However, most calculated N_e values were small (100 individuals or less). Only three sample sites (KO, AN, and PW) showed consistently high values for N_e (500 or higher, or infinite) for most methods.

Discussion

The analyses presented here provide valuable information for the conservation management of the Endangered European Tree Frog species, *Hyla arborea*, which suffers from population isolation in its northern distribution range. In Lower Saxony, a weak correlation between genetic and geographic distances suggests a low level of recent gene flow among localities, and further analyses indicate a lack of current migration at least during the last generation. Two major genetic clusters, one in the east and one in the west, were found with some admixture in a central population. Both main clusters were further subdivided into several distinct regional clusters. The substantial population structure, verified by significant genetic distances among localities, suggests that the populations are currently isolated to a large extent. Consequently, conservation management is needed to ensure the long-term persistence of this species in Lower Saxony with suitable effective population sizes and high levels of genetic diversity that are necessary to counteract the reductions in fitness and adaptive potential (Andersen et al. 2004; Frankham 2005; Allentoft and O'Brien 2010; Angelone 2010).

Isolation by Geographic and Genetic Distances

In addition to the Mantel test, the landscape genetic analysis in GESTE provided some insight into the role of geographic distance on genetic differentiation. While the models including latitude and longitude alone did not offer a better explanation for genetic differentiation than the null model, the most complex model including the interactions between latitude and longitude did. We interpret this as the effect of the geographic distance

Table 4. Results of BIMr analysis (means of posterior probability and Alpha for run 1) for estimating migration rates among localities with European Tree Frogs in Lower Saxony. The factor G1 is the geographic distance. Alpha 0 and Alpha 1 represent estimates of the constant term and factor G1, respectively.

Model	Factor	Posterior probability	Alpha 0	Alpha 1
Model 0		0.79	1.64	
Model 1	G1 = geographic distance	0.21	1.48	0.14

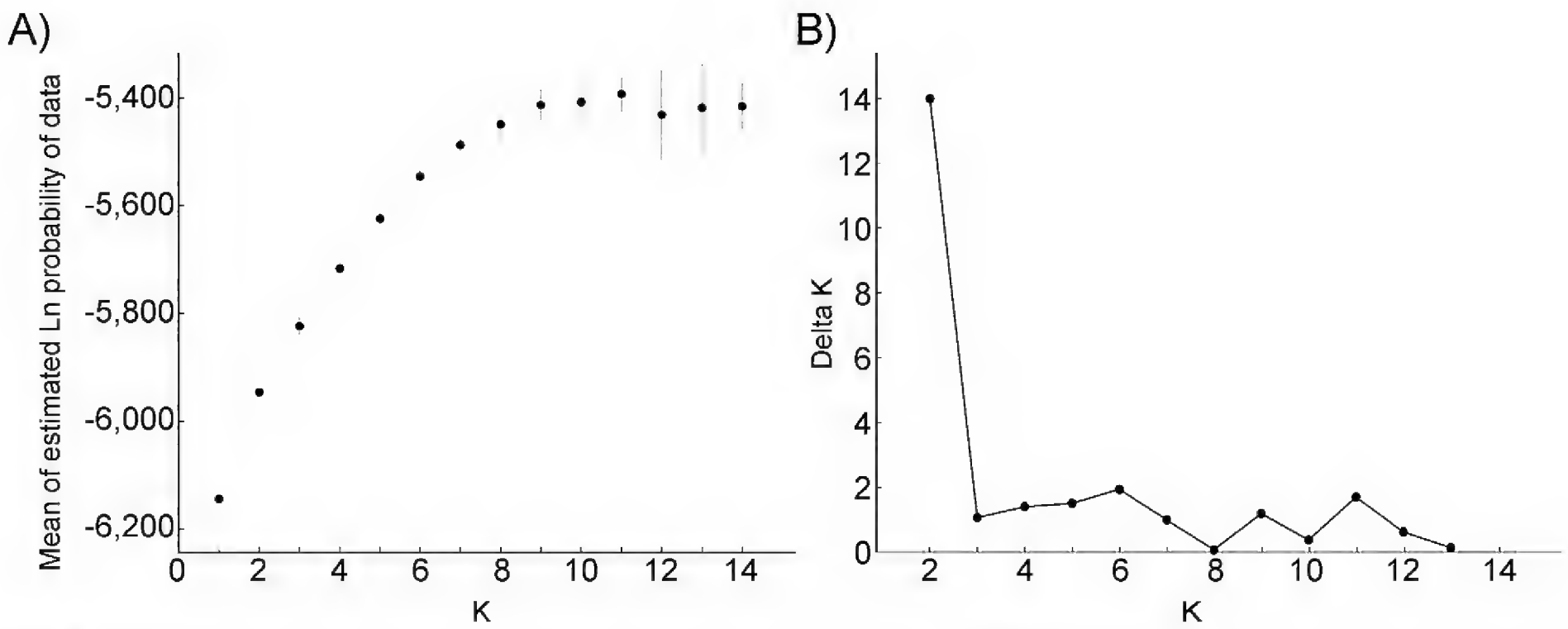

Fig. 4. Mean values of estimated Ln probability of data (LnPD) for each *K* (a) and delta *K* (b) when prior population information was implemented.

on genetic dissimilarity between localities. Overall, however, the posterior probability and alpha values illustrate that the distance effect is not very large. Habitat fragmentation might play a more important role in shaping the genetic structure of the tree frogs in this area. According to Podloucky and Fischer (2013), habitat fragmentation in Lower Saxony is mainly caused by the loss of summer habitat, breeding ponds, and corridors suitable for migration. This is in accordance with the disconnected distribution (Fig. 1) and significant genetic distances (F_{ST} and D_{est}) among most tree frog populations.

A significant population structure as a result of limited dispersal between isolated populations is typically accompanied by a slight to moderate effect of isolation by distance. Isolation by distance was detected in some European amphibian species (*Rana dalmatina*, Sarasola-Puente et al. 2012; *Bombina variegata*: Weihmann et al. 2009; Hantzschmann et al. 2020), but not others (*Bufo calamita*: Allentoft et al. 2009; *Bombina bombina*: Dolgener et al. 2012). For the tree frogs in Lower Saxony, the small positive correlation ($r \sim 0.28$) between genetic and geographic distances suggests a very low level of recent gene flow among localities. This finding is in accordance with earlier studies on tree frogs, which reported small to moderate correlation coefficients between both distances and significant population structure (Andersen et al. 2004; Angelone and Holderegger 2009; Arens et al. 2006). In all these studies, limited gene flow was explained by habitat fragmentation, particularly the loss of breeding ponds. In contrast, in those frog species which occur in more continuous habitats or that have higher dispersal capacities, the

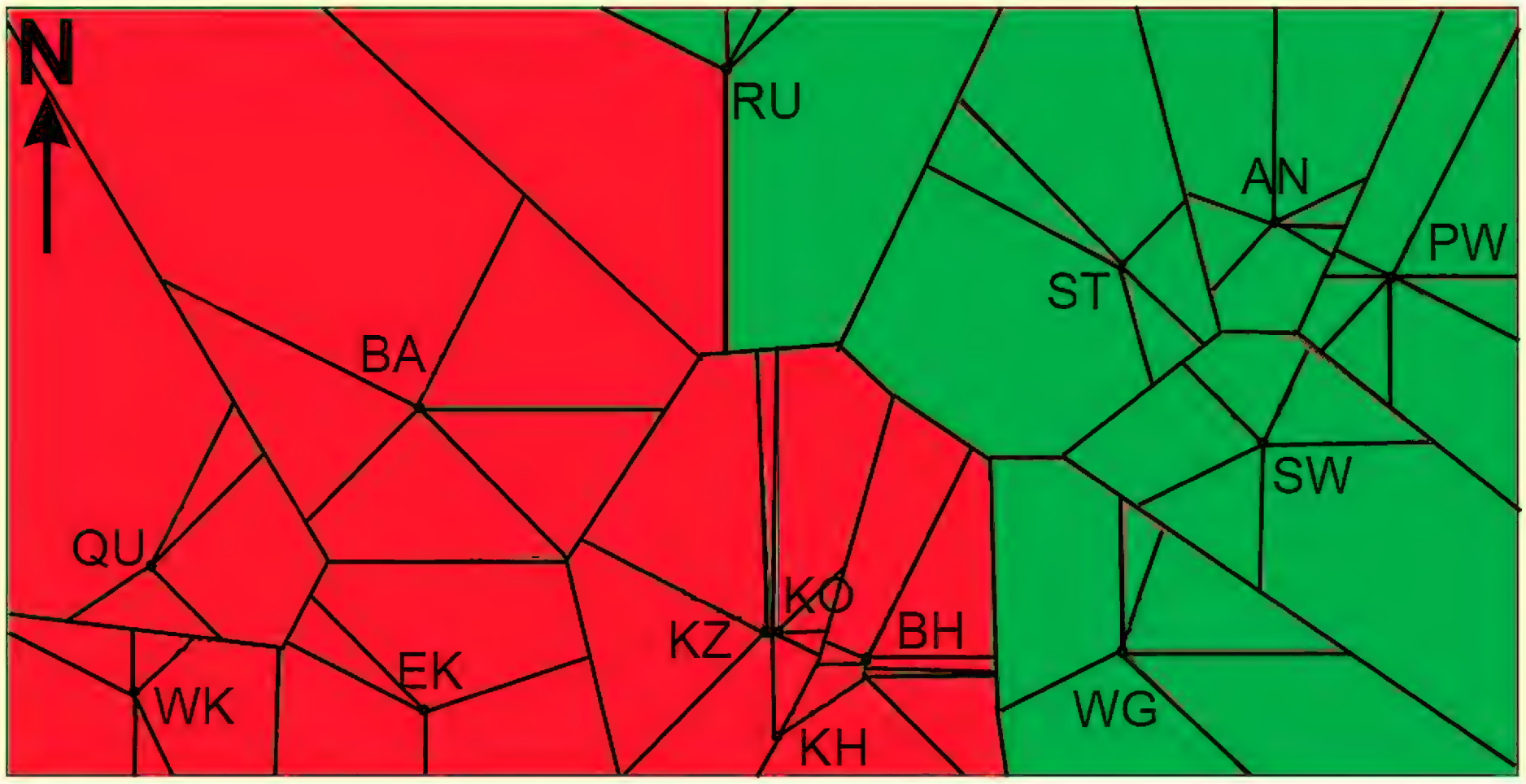

Fig. 5. Map of geographic-genetic cluster membership for $K_{max} = 2$ as inferred by TESS.

Table 5. Results for effective populations sizes (N_e) calculated with the N_e Estimator for three different methods and different P_{Crit} values. N_e values were the same for P_{Crit} = 0.02, 0.01, and 0+. Only 0+ is used in the Molecular Coancestry method. Mean values (N_e) and Confidence Intervals (CIs) are given for each sample site, method, and P_{Crit} option.

		The three methods				
		Linkage disequilibrium		Heterozygote excess		Molecular coancestry
Sample site	P_{Crit}	0.05	0.02/0.01/0+	0.05	0.02/0.01/0+	0+
QU	N_e	55.7	55.7	7.8	7.8	9.5
	CIs	1.4 – inf.	1.4 – inf.	3.7 – inf.	3.7 – inf.	5.1 – 15.2
WK	N_e	1.3	1.3	5.1	5.1	2.7
	CIs	0.7 – 2.7	0.7 – 2.7	2.5 – inf.	2.5 – inf.	2.0 – 3.5
EK	N_e	16.3	33.5	9.9	9.9	8.2
	CIs	5.6 – inf.	8.7 – inf.	4.5 – inf.	4.5 – inf.	2.7 – 16.9
KZ	N_e	46.3	28.2	34.7	34.7	5.6
	CIs	15.8 – inf.	12.1 – 319.2	7.5 – inf.	7.5 – inf.	2.3 – 10.5
KO	N_e	inf.	611.3	251.5	251.5	inf.
	CIs	11.4 – inf.	10.4 – inf.	4.3 – inf.	4.3 – inf.	inf. – inf.
KH	N_e	319.3	159.7	inf.	44.3	5.7
	CIs	30.5 – inf.	30.2 – inf.	5.8 – inf.	6.0 – inf.	3.2 – 8.9
BH	N_e	83.5	73.5	731.7	inf.	inf.
	CIs	21.0 – inf.	21.3 – inf.	7.2 – inf.	7.7 – inf.	inf. – inf.
BA	N_e	177.2	188.0	322.0	322.0	10.8
	CIs	26.5 – inf.	26.8. inf.	6.5 – inf.	6.5 – inf.	1.8 – 27.7
RU	N_e	19.3	27.5	67.7	81.9	inf.
	CIs	8.4 – 106.4	10.8 – inf.	6.8 – inf.	7.1 – inf.	inf.
WG	N_e	31.1	65.5	inf.	inf.	9.1
	CIs	16.0 – 113.5	26.0 – inf.	8.1 – inf.	9.3 – inf.	3.8 – 16.6
ST	N_e	34.7	77.5	39.7	51.0	inf.
	CIs	15.7 – 315.7	25.4 – inf.	6.4 – inf.	7.2 – inf.	inf. – inf.
AN	N_e	inf.	inf.	inf.	inf.	inf.
	CIs	41.7 – inf.	74.0 – inf.	30.9 – inf.	57.8 – inf.	inf. – inf.
SW	N_e	37.3	36.0	inf.	inf.	43.4
	CIs	15.2 – inf.	16.1 – inf.	inf. – inf.	280 – inf.	0 – 217.7
PW	N_e	631.9	inf.	inf.	inf.	inf.
	CIs	36.2 – inf.	73.1 – inf.	6.1 – inf.	6.6 – inf.	inf. – inf.

correlation between geographic and genetic distance is higher (e.g., Pröhl et al. 2006) or disappears in case of panmixia (Leblois et al. 2000).

The BIMr analysis revealed that contemporary migration (i.e., during the last generation) was practically absent among the sample sites of tree frogs in Lower Saxony. This is in contrast to the migration rates calculated with BayesAss and the results of the Structure analysis (see below), which imply that individuals of mixed ancestry exist in the different genetic clusters. However, gene flow over the last few tree frog generations may have suffered due to the expanding habitat fragmentation, while some decades ago far more tree frog localities were reported for lower Saxony, thus, the connectivity among them was much better (Manzke and Podloucky 1995). Therefore, the signals for gene flow are still apparent in the results of some analyses (Structure, BayesAss) but not in those where the calculations are restricted to the most recent years (BIMr).

Genetic Diversity

The expected microsatellite heterozygosity (H_e) has been measured in a number of previous population genetic studies of the European Tree Frog. Interestingly, H_e was higher in the current study area (H_e: 0.60 – 0.79) than in most other northern areas where the genetic situation of the tree frog was investigated (e.g., H_e values in Denmark: 0.35–0.54, Andersen et al. 2004; Switzerland: 0.27–0.71, Angelone and Holderegger 2009; and the Netherlands:

0.39–0.59, Arens et al. 2006). As expected, the more peripheral populations, such as those in Denmark and the Netherlands, show lower genetic diversity values. In comparison, the *mt*haplotypes diversity (mean $h = 0.38$, $n = 14$ populations) is lower in the Lower Saxony area than in the southern part of the distribution range (Greece, Albania, Croatia, Serbia, and Romania) were the average h amounts to 0.7 ($n = 20$ populations, calculated from Dufresnes et al. 2013, Table S2). From all cumulative data within the framework of this study, we can conclude that in central and northern Europe, human induced fragmentation processes involving habitat destruction in a previously widely distributed frog species are contributing to the depletion of genetic diversity.

Genetic Structure and Conservation Units

The Bayesian cluster analyses conducted with STRUCTURE and TESS support the division of the tree frog populations into two major geographic-genetic clusters, one in the west and one in the east. One population in the south-east of the area (WG) shows admixture between the two groups and therefore a relatively high genetic diversity. Thus, it seems that both groups were previously connected by migrating animals when habitat fragmentation was less severe. This result is consistent with the moderate correlation between geographic distance and genetic distances. Both analyses also provide evidence for further fragmentation within both groups. In contrast, the haplotype network does not indicate any older, distinct (e.g., postglacial) lineages supporting the finding of Dufresnes et al. (2013), that only one evolutionary unit is present in this area.

Microsatellite pairwise D_{est} and F_{ST} values all showed significant genetic differentiation except for the two closest sites (KZ and KO) in the West of Hannover. However, *mt*haplotype distribution and Bayesian analyses of the microsatellites suggest distinct relationships among the currently fragmented localities. In the Northeast of Lower Saxony, the distribution of the *mt*haplotypes indicates a former connection of the populations along the river Elbe. Interestingly, the easternmost occurrences at AN, PW, and SW in the current and former distributions display a relatively well-connected area, nonetheless the presently distinct genetic sub-clusters in the Bayesian analyses and pairwise F_{ST} values are relatively high. The significant F_{IS} value found for SW indicates that the separation of this site may have resulted in inbreeding in an isolated population. Altogether, the available microsatellite data point to recent fragmentation of tree frog populations in this area, while similar *mt*haplotypes provide some evidence for a former connection.

Interestingly, there is a significant genetic divergence between the sample sites KZ and KO in the West and the sample sites KH and BH in the East of Hannover. One possible explanation is that recently constructed motorways in combination with genetic drift contributed to population differentiations, which are also apparent in the *mt*haplotype frequencies. Roads have been identified as barriers to gene flow in some other amphibians (Arens et al. 2007; Lesbarrères et al. 2006). One central question is whether these relatively young barriers (motorways expanding in the 1960s, and dense urban areas) are the only reason for the differentiation of these formerly linked tree frog localities (Manzke and Podloucky 1995). The low haplotype diversity in KZ, EK, and QU points to a loss of genetic diversity as a consequence of increased genetic drift in isolated occurrences.

In summary, the genetic analyses point to a highly structured population, as was observed in other surveys of European amphibians (Dolgener et al. 2012; Rowe and Beebee 2007; Sarasola-Puente et al. 2012; Hantzmann et al. 2020). The risks of fragmentation include population reduction, loss of genetic diversity and declining fitness, and finally extinction (Hitchings and Beebee 1997; Cushman 2006). To reverse such negative processes, conservation management that takes the genetic population structure into account is important (Allentoft et al. 2009; Olsen et al. 2014). For this endangered and fragmented frog species, we suggest delineating the two major genetic clusters as conservation units; and then within those, intense reconnection efforts should be undertaken by creating suitable habitats for migrating frogs. Moreover, there is clear evidence of admixture in WG, and gene flow along this route could be reestablished between both clusters.

Conservation Measures

In our opinion, future conservation management should be directed towards two aims. The first aim is to maintain high genetic diversity in large and stable populations within each conservation unit. In this context, it has been argued that an effective breeding size (N_e) of at least 50 animals is necessary to avoid inbreeding in the short term and that an N_e of 500 is necessary to maintain the evolutionary potential that would allow adaptations to environmental changes and assure long term viability (Jamieson and Allendorf 2012). Most of our isolated sample sites (e.g., BA, Fig. 3B–C) or sub-clusters (e.g., KH-BH) do not reach these effective population sizes. For tree frogs, the ratio of effective breeding size (N_e) to census size (N) is ~ 0.5 (Broquet et al. 2009). Therefore, we recommend the monitoring of population sizes and maintaining population sizes of at least 100 breeding frogs in isolated populations, i.e., each sub-cluster, for short term conservation goals, but increasing the population sizes to 1,000 or more embedded in each of several meta-population systems in every conservation unit (see also Andersen et al. 2004; Frankham et al. 2014). In cases where populations within a conservation unit are genetically and geographically separated, genetic rescue can be attained by establishing corridors to stimulate dispersal (e.g., in AN-SW-PW). Dense networks of suitable spawning

ponds have been destroyed by habitat conversion but are of great importance for the maintenance of large tree frog populations and the connection of subpopulations. There are several reports that tree frogs not only respond well to new suitable water bodies, but also depend on them for migrations exceeding several km and often colonize them in subsequent breeding seasons (e.g., Angelone and Holderegger 2009; Brandt and Lüers 2017; Hansen 2004; Schwartze 2002; Zumbach 2004).

The second aim is to maintain overall genetic diversity among the genetic clusters within the species and to protect local co-adapted gene complexes (Savolainen et al. 2013). To achieve this second goal, we recommend the re-establishment of gene flow between genetic clusters where possible, but at a lower level than within them. This particularly applies to the western and eastern clusters between which (former) gene flow is evidenced by the STRUCTURE analysis (Fig. 3). Habitat reconnection between these areas would allow a few frogs to travel between the breeding ponds of different clusters, thereby refreshing genetic diversity and counterbalancing the loss of genetic diversity through drift, while diverse selection pressures would sustain local adaptation. Levels of genetic diversity inferred from neutral markers are not necessarily correlated with variation in locally adapted traits. In this context, more research is necessary to understand which traits are locally adapted and how their variation affects the fitness of a population. Very isolated and small populations might benefit from translocations, i.e., the introduction of individuals from other populations. In such cases, translocations should be restricted to within the conservation unit to avoid causing outbreeding depressions that have sometimes been observed between distantly related populations (Sagvik et al. 2005).

Conclusions

Populations of the European Tree Frog in Lower Saxony are highly fragmented geographically and genetically, and therefore endangered. We identified two major genetic clusters and recommend that they should be considered as local conservation units. Conservation efforts should entail a reconnection of the populations within these conservation units, and to a lesser degree between them. Moreover, the maintenance of large and stable meta-populations within genetic sub-clusters (mostly consisting of isolated populations) needs to be achieved for long-term survival. For translocations of individuals to recovering very small and inbred populations or for reintroduction, we suggest a mixing of individuals from different populations within the same conservation unit to increase genetic diversity and enhance the adaptive capacity regarding changing environmental conditions. This study offers one example of how population genetic studies can help to delineate conservation units, and our recommendations might apply just as well to other endangered species where declines are connected to increasing habitat fragmentation.

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Literature Cited

Allentoft ME, O'Brien J. 2010. Global amphibian declines, loss of genetic diversity, and fitness: a review. *Diversity* 2: 47–71.

Allentoft ME, Siegismund HR, Briggs L, Andersen LW. 2009. Microsatellite analysis of the Natterjack Toad (*Bufo calamita*) in Denmark: populations are islands in a fragmented landscape. *Conservervation Genetics* 10: 15–28.

Andersen LW, Fog K, Damgaard C. 2004. Habitat fragmentation causes bottlenecks and inbreeding in the European Tree Frog (*Hyla arborea*). *Proceedings of the Royal Society of London B-Biological Sciences* 271: 1,293–1,302.

Angelone S. 2010. Are differences in fitness traits related to genetic cluster? An empirical test on the European Tree Frog. *Biological Conservation* 143: 471–478.

Angelone S, Holderegger R. 2009. Population genetics suggests effectiveness of habitat connectivity measures for the European Tree Frog in Switzerland. *Journal of Applied Ecology* 46: 879–887.

Arens P, van't Westende W, Bugter R, Smulders MJM, Vosman B. 2000. Microsatellite markers for the European Tree Frog, *Hyla arborea*. *Molecular Ecology* 9: 1,944–1,946.

Arens P, Bugter R, van't Westende W, Zollinger R, Stronks J, Vos CC, Smulders MJM. 2006. Microsatellite variation and population structure of a recovering Tree Frog (*Hyla arborea* L.) metapopulation. *Conservation Genetics* 7: 825–835.

Arens P, van der Sluis T, van't Westende W, Vosman B, Vos CC, Smulders MJM. 2007. Genetic population differentiation and connectivity among fragmented Moor Frog (*Rana arvalis*) populations in The Netherlands. *Landscape Ecology* 22: 1,489–1,500.

Balkenhol N, Waits LP, Dezzani RJ. 2009. Statistical approaches in landscape genetics: an evaluation of methods for linking landscape and genetic data.

Ecography 32: 818–830.
Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F. 2004. Genetix 4.05, population genetics software for Windows™. Université de Montpellier II, Montpellier, France.
Brandt T, Lüers E. 2017. Die erfolgreiche Wiederansiedlung von Europäischen Laubfröschen (*Hyla arborea*) in der Sachsenhäger Niederung, Landkreis Schaumburg, Niedersachsen. *Zeitschrift für Feldherpetologie* 24: 209–222.
Broquet T, Berset-Braendli L, Emaresi G, Fumagalli L. 2007. Buccal swabs allow efficient and reliable microsatellite genotyping in amphibians. *Conservervation Genetics* 8: 509–511.
Broquet T, Jaquiery J, Perrin N. 2009. Opportunity for sexual selection and effective population size in the lek-breeding European Treefrog (*Hyla arborea*). *Evolution* 63: 674–683.
Buschmann H, Scheel B, Brandt T. 2006. *Amphibien und Reptilien im Schaumburger Land und am Steinhuder Meer.* Natur und Text, Rangsdorf, Germany. 184 p.
Chen C, Durand E, Forbes F, François O. 2007. Bayesian clustering algorithms ascertaining spatial population structure: a new computer program and a comparison study. *Molecular Ecology Notes* 7: 747–756.
Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1,657–1,659.
Crandall KA, Bininda-Emonds OR, Mace GM, Wayne RK. 2000. Considering evolutionary processes in conservation biology. *Trends in Ecology and Evolution* 15: 290–295.
Crnokrak P, Roff DA. 1999. Inbreeding depression in the wild. *Heredity* 83: 260–270.
Cushman SA. 2006. Effects of habitat loss and fragmentation on amphibians: a review and prospectus. *Biological Conservation* 128: 231–240.
Dolgener N, Schröder C, Schneeweiss N, Tiedemann R. 2012. Genetic population structure of the Fire-bellied Toad, *Bombina bombina,* in an area of high population density: implications for conservation. *Hydrobiologia* 689: 111–120.
Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ, Ovenden JR. 2014. NeEstimator V2: re-implementation of software for the estimation of contemporary effective population size (N_e) from genetic data. *Molecular Ecology Resources* 14: 209–214.
Dubey S, Ursenbacher S, Pellet J, Fumagalli L. 2009. Genetic differentiation in two European Tree Frog (*Hyla arborea*) metapopulations in contrasted landscapes of western Switzerland. *Amphibia-Reptilia* 30: 127–133.
Dufresnes C, Wassef J, Ghali K, Brelsford A, Stöck M, Lymberakis P, Crnobrnja-Isailovic J, Perrin N. 2013. Conservation phylogeography: Does historical diversity contribute to regional vulnerability in European Tree Frogs (*Hyla arborea*)? *Molecular Ecology* 22: 5,669–5,684.
Earl DA, von Holdt BM. 2012. Structure Harvester: a website and program for visualizing Structure output and implementing the Evanno method. *Conservation Genetics Resources* 4: 359–361.
Edenhamn P, Höggren M, Carlson A. 2000. Genetic diversity and fitness in peripheral and central populations of the European Tree Frog, *Hyla arborea*. *Hereditas* 133: 115–122.
Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1,792–1,797.
Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. *Molecular Ecology* 14: 2,611–2,620.
Excoffier L, Laval G, Schneider S. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics* 1: 47–50.
Fahrig L, Merriam G. 1994. Conservation of fragmented populations. *Conservation Biology* 8: 50–59.
Faubet P, Waples RS, Gaggiotti OE. 2007. Evaluating the performance of a multilocus Bayesian method for the estimation of migration rates. *Molecular Ecology* 16: 1,149–1,166.
Faubet P, Gaggiotti OE. 2008. A new Bayesian method to identify the environmental factors that influence recent migration. *Genetics* 178: 1,491–1,504.
Foll M, Gaggiotti O. 2006. Identifying the environmental factors that determine the genetic structure of populations. *Genetics* 174: 875–891.
François O, Ancelet S, Guillot G. 2006. Bayesian clustering using hidden Markov random fields in spatial population genetics. *Genetics* 174: 805–816.
Frankham R. 2005. Genetics and extinction. *Biological Conservation* 126: 131–140.
Frankham R, Bradshaw CJA, Brook BW. 2014. Genetics in conservation management: revised recommendations for the 50/500 rules, Red List criteria, and population viability analyses. *Biological Conservation* 170: 56–63.
Gerlach G, Jueterbock A, Kraemer P, Deppermann J, Harmand P. 2010. Calculations of population differentiation based on G(ST) and D: forget G(ST) but not all of statistics! *Molecular Ecology* 19: 3,845–3,852.
Goebel AM, Donnelly JM, Atz ME. 1999. PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase I, and cytochrome b in bufonids and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. *Molecular Phylogenetics and Evolution* 11: 163–199.
Goudet J. 1995. Fstat (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity* 86: 485–

486.
Green PJ. 1995. Reversible jump Markov chain Monte Carlo computation and Bayesian model determination. *Biometrika* 82: 711–732.
Guo SW, Thompson EA. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 48: 361–372.
Hansen F. 2004. Verbreitung und Gefährdung des Laubfrosches (*Hyla arborea*) auf Bornholm (Dänemark) und Maßnahmen zur Lebensraumoptimierung. *Zeitschrift für Feldherpetologie* (*Supplement* 5): 133–143.
Hantzschmann AL, Sinsch U, Göttlicher C, Pröhl H. 2020. Conservation genetics of Yellow-bellied Toads (*Bombina variegata*): a matter of geographical scale and isolation. *Conservation Genetics* 22: 83–96.
Hedrick PW, Kalinowski ST. 2000. Inbreeding depression in conservation biology. *Annual Review of Ecology and Systematics* 25(31): 139–162.
Hitchings SP, Beebee TJ. 1997. Genetic substructuring as a result of barriers to gene flow in urban *Rana temporaria* (Common Frog) populations: implications for biodiversity conservation. *Heredity* 79: 117–127.
Jamieson IG, Allendorf FW. 2012. How does the 50/500 rule apply to MVPs? *Trends in Ecology and Evolution* 27: 578–584.
Jenness J. 2005. Distance Matrix (dist_mat_jen.avx) extension for ArcView 3.x, v. 2. Jenness Enterprises, Flagstaff, Arizona, USA. Available: http://www.jennessent.com/arcview/dist_matrix.htm [Accessed: 1 August 2011].
Jensen JL, Bohonak AJ, Kelley ST. 2005. Isolation by distance, web service. *BMC Genetics* 6: 6.
Jost L. 2008. G(ST) and its relatives do not measure differentiation. *Molecular Ecology* 17: 4,015–4,026.
Krug A, Pröhl H. 2013. Population genetics in a fragmented population of the European Tree Frog (*Hyla arborea*). *Amphibia-Reptilia* 34: 95–107.
LANUV. 2011. Laubfrosch-*Hyla arborea*. Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, German. Available: http://www.lanuv.nrw.de [Accessed: 1 December 2011].
Leblois R, Rousset F, Tikel D, Moritz C, Estoup A. 2000. Absence of evidence for isolation by distance in an expanding Cane Toad (*Bufo marinus*) population: an individual-based analysis of microsatellite genotypes. *Molecular Ecology* 9: 1,905–1,909.
Lesbarrères D, Primmer CR, Lode T, Merila J. 2006. The effects of 20 years of highway presence on the genetic structure of *Rana dalmatina* populations. *Ecoscience* 13: 531–538.
Luquet E, David P, Lena JP, Joly P, Konecny L, Dufresnes C, Perrin N, Plénet S. 2011. Heterozygosity-fitness correlations among wild populations of European Tree Frog (*Hyla arborea*) detect fixation load. *Molecular Ecology* 20: 1,877–1,887.
Madeira F, Pearce M, Tivey ARN, Basutkar P, Lee J, Edbali O, Madhusoodanan N, Kolesnikov A, Lopez R. 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Research* 50: W276–W 279.
Manzke U, Podloucky R. 1995. Der Laubfrosch *Hyla arborea* in Niedersachsen und Bremen–Verbreitung, Lebensraum, Bestandsituation. Pp. 57–72 In: *Der Laubfrosch* (*Hyla arborea*)*, Ökologie und Artenschutz.* Editor, Geiger A. Mertensiella, Bonn, Germany. 196 p.
Meyer F, Buschendorf J, Zuppke U, Braumann F, Schädler M, Grosse WR. 2004. *Die Lurche und Kriechtiere Sachsen-Anhalts. Verbreitung, Ökologie, Gefährdung, und Schutz.* Laurenti-Verlag, Bielefeld, Germany. 240 p.
Moritz C, Schneider CJ, Wake DB. 1992. Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Systematic Biology* 41: 273–291.
Moritz C. 1994. Defining evolutionarily significant units for conservation. *Trends in Ecology and Evolution* 9: 373–374.
Nei M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York, New York, USA. 512 p.
NLWKN. 2011. *Vollzugshinweise zum Schutz von Amphibien und Reptilienarten in Niedersachsen Hannover.* Niedersächsischer Landesbetrieb für Wasserwirtschaft, Küstenund Naturschutz, Hannover, Germany. 33 p.
Olsen MT, Andersen LW, Dietz R, Teilmann J, Härkönen T, Siegismund HR. 2014. Integrating genetic data and population viability analyses for the identification of Harbour Seal (*Phoca vitulina*) populations and management units. *Molecular Ecology* 23: 815–831.
Palsbøll PJ, Berube M, Allendorf FW. 2007. Identification of management units using population genetic data. *Trends in Ecology and Evolution* 22: 11–16.
Pimm S, Raven P. 2000. Biodiversity: extinction by numbers. *Nature* 403: 843–845.
Podloucky R, Fischer C. 2013. *Rote Listen und Gesamtlisten der Amphibien und Reptilien in Niedersachsen und Bremen. Informationsdienst Naturschutz Niedersachsen, Band 4.* Niedersächsischer Landesbetrieb für Wasserwirtschaft, Küstenund Naturschutz, Hannover, Germany. 167 p.
Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.
Pröhl H, Koshy RA, Mueller U, Rand AS, Ryan MJ. 2006. Geographic variation of genetic and behavioral traits in northern and southern Túngara Frogs. *Evolution* 60: 1,669–1,679.
Rannala B. 2007. *BayesAss Edition 3.0 User's Manual, Update 2015*. University of California Davis, Davis, California, USA. 12 p.
Richter M, Mügge G. 2012. Bestandsentwicklung

und Schutz des Laubfroschs (*Hyla arborea*) im Landkreis Diepholz/Niedersachsen. *Zeitschrift für Feldherpetologie* 19: 79–90.

Rousset F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* 145: 1,219–1,228.

Rousset F. 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

Rowe G, Beebee T. 2007. Defining population boundaries: use of three Bayesian approaches with microsatellite data from British Natterjack Toads (*Bufo calamita*). *Molecular Ecology* 16: 785–796.

Sagvik J, Uller T, Olsson M. 2005. Outbreeding depression in the Common Frog, *Rana temporaria*. *Conservation Genetics* 6: 205–211.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning*. Cold Spring Harbor Laboratory Press, New York, New York, USA. 1,659 p.

Sarasola-Puente V, Madeira MJ, Gosá A, Lizana, M, Gómez-Moliner B. 2012. Population structure and genetic diversity of *Rana dalmatina* in the Iberian Peninsula. *Conservation Genetics* 13: 197–209.

Savolainen O, Lascoux M, Merilä J. 2013. Ecological genomics of local adaptation. *Nature Reviews Genetics* 14: 807–820.

Schwartze M. 2002. Neuanlage und Verbesserungen von Kleingewässern für den Laubfrosch und andere Amphibien: eine Untersuchung im östlichen Münsterland (NRW). *Zeitschrift für Feldherpetologie* 9: 61–73.

Selkoe KA, Toonen RJ. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* 9: 615–629.

Shaffer HB, Gidiş M, McCartney-Melstad E, Neal KM, Oyamaguchi HM, Tellez M, Toffelmier EM. 2015. Conservation genetics and genomics of amphibians and reptiles. *Annual Reviews of Animal Biosciences* 3: 113–138.

Sih A, Jonsson BG, Luikar G. 2000. Habitat loss: ecological, evolutionary, and genetic consequences. *Trends in Ecology and Evolution* 15: 132–134.

Smith, MA, Green DM. 2006. Sex, isolation, and fidelity: unbiased long-distance dispersal in a terrestrial amphibian. *Ecography* 29: 649–658.

Spear SF, Peterson CR, Matocq MD, Storfer A. 2005. Landscape genetics of the Blotched Tiger Salamander (*Ambystoma tigrinum melanostictum*). *Molecular Ecology* 14: 2,553–2,564.

Stöck M, Dufresnes C, Litvinchuk SN, Lymberakis P, Biollay S, Berroneau M, Borzée A, Ghali K, Ogielska M, Perrin N. 2012. Cryptic diversity among Western Palearctic tree frogs: postglacial range expansion, range limits, and secondary contacts of three European Tree Frog lineages (*Hyla arborea* group). *Molecular Phylogenetics and Evolution* 65: 1–9.

Storfer A, Murphy MA, Spear SF, Holderegger R, Waits LP. 2010. Landscape genetics: Where are we now? *Molecular Ecology* 19: 3,496–3,514.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues AS, Fischman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science* 306: 1,783–1,786.

Tamura K, Nei M, Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America* 101: 11,030–11,035.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1,596–1,599.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.

Vergara M, Basto MP, Madeira MJ, Gómez-Moliner BJ, Santos-Reis M, Fernandes C, Ruiz-González A. 2015. Inferring population genetic structure in widely and continuously distributed carnivores: the Stone Marten (*Martes foina*) as a case study. *PLoS One* 10: e0134257.

Weihmann F, Podloucky R, Hauswaldt S, Pröhl H. 2009. Naturschutzgenetische Untersuchungen von Populationen der Gelbbauchunke (*Bombina v. variegata*) im südlichen Niedersachsen. *Zeitschrift für Feldherpetologie* 16: 183–200.

Weir BS, Cockerham CC. 1984. Estimating F-statistics for the analysis of population-structure. *Evolution* 38: 1,358–1,370.

Wright S. 1943. Isolation by distance. *Genetics* 28: 114.

Zumbach S. 2004. Die Laubfrösche (*Hyla arborea* und *Hyla intermedia*) in der Schweiz – Verbreitung, Gefährdung, und Schutz. *Zeitschrift für Feldherpetologie* (Supplement 5): 183–192.

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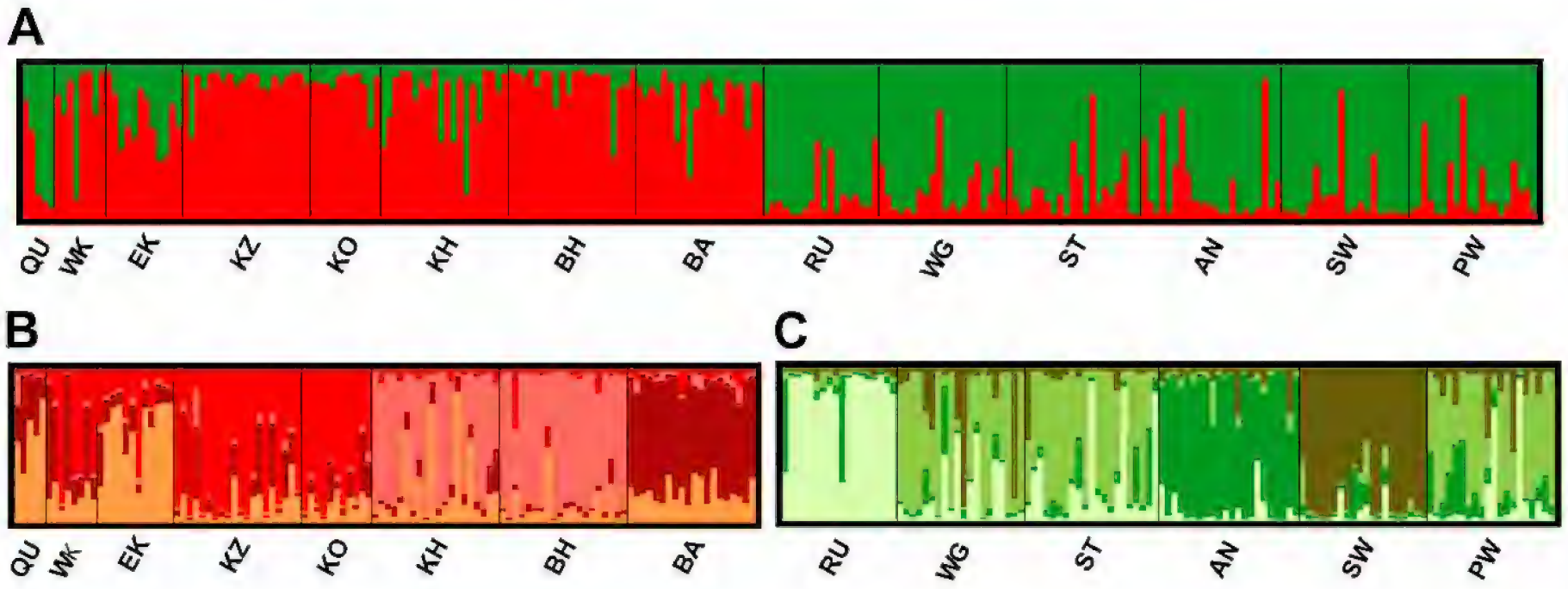
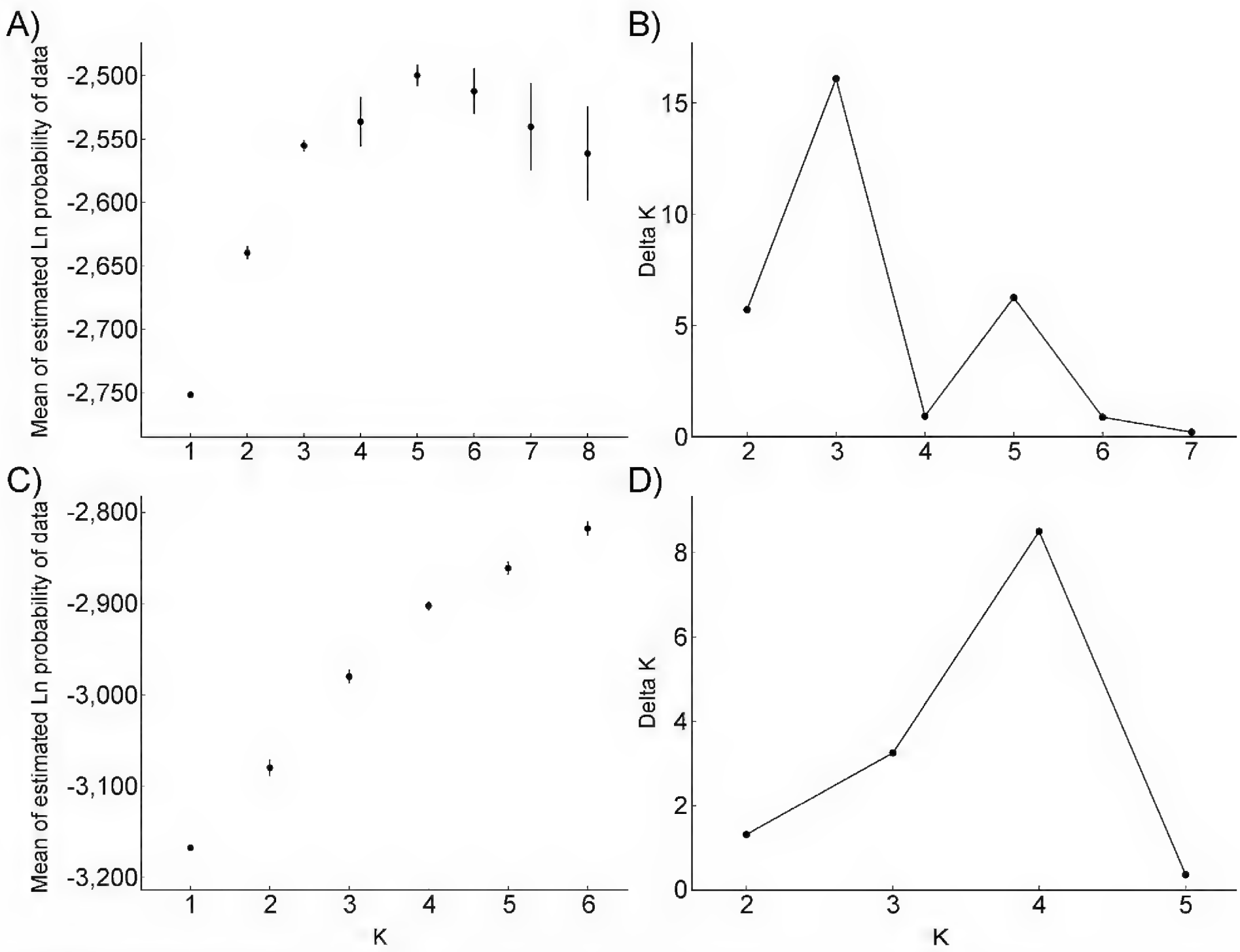
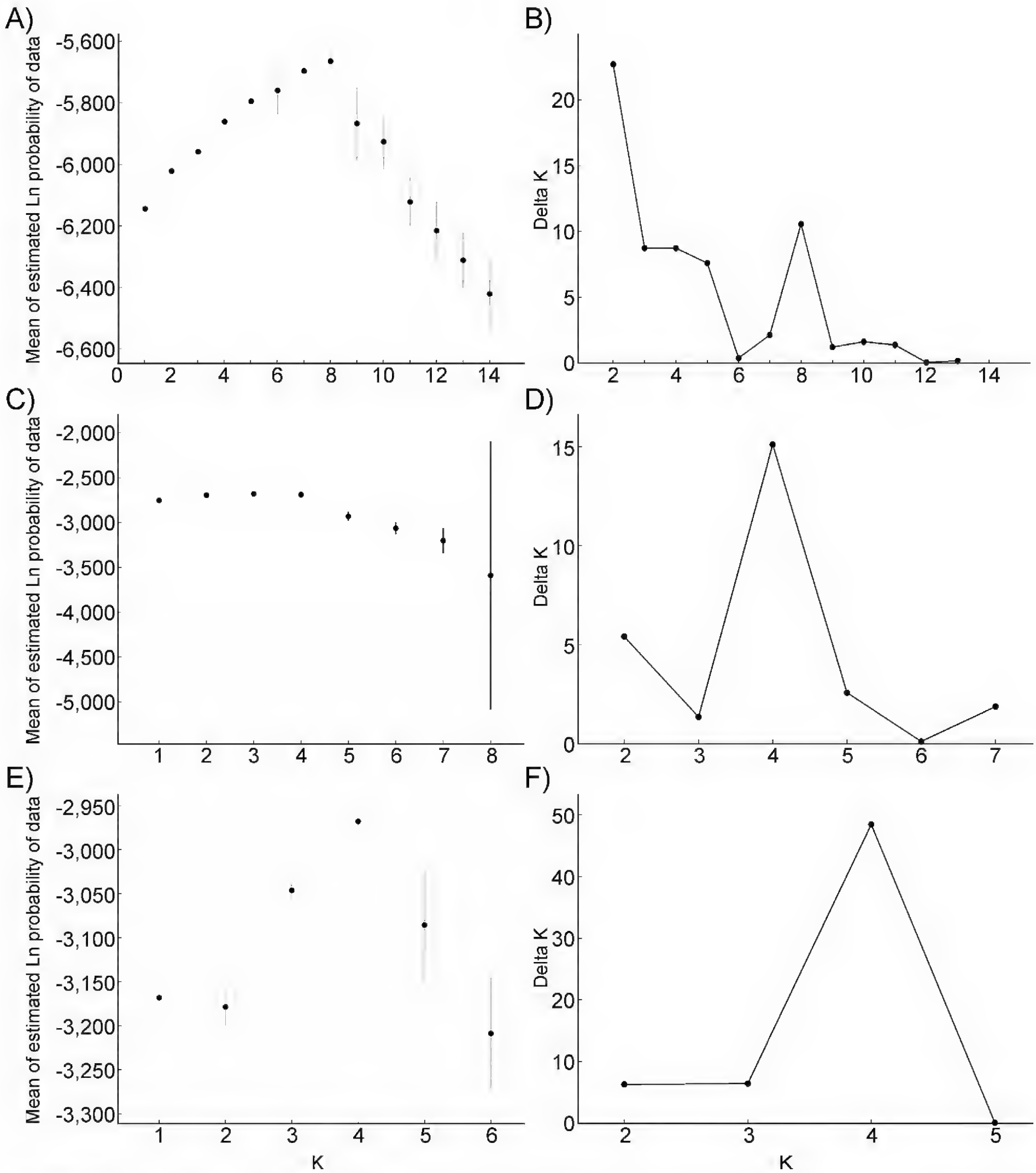
Heike Pröhl discovered her interest in studying frogs in the wild while she studied Biology at the University of Hannover, and while spending a year studying tropical biology at the Universidad de Costa Rica. During that appointment, she benefitted from a wide range of field courses, including herpetology. After sampling field data on Neotropical frog behavior and ecology for her Diploma and Doctoral theses, as well as her Postdoctoral project, she started to work as a Junior Professor at the Veterinary University of Hannover (TiHo) in Germany. Heike is currently an apl. Professor for Zoology and teaches courses related to Zoology, Ecology, and Evolution to biology and veterinary students. Her research focuses on the behavior, ecology, and conservation of Neotropical and European amphibians.



Supplementary Fig. S1. Estimation of the number of *Hyla arborea* populations using the program STRUCTURE ver. 2.3.1 (Pritchard et al. 2000) without prior population information; QU, WK, EK, etc. = sample sites, separated by fine black lines. Each individual is represented by a single vertical line broken into *K*-colored segments, with lengths proportional to each of the *K*-inferred clusters. (A) Plot for $K = 2$ in the analysis of the entire data set, (B) plot for $K = 4$, and (C) $K = 4$ for hierarchical analysis on each of the two main clusters.



Supplementary Fig. S2. Mean values of estimated Ln probability of data (LnPD) for each *K* (A, C) and delta *K* (B, D) calculated from STRUCTURE runs with STRUCTURE HARVESTER (20 replicates per *K*) in those analyses where prior population information was implemented. (A–B), graphs for hierarchical analysis of the red cluster; (C–D), corresponding graphs for the green cluster (compare to Fig. 3 in the main text).



Supplementary Fig. S3. Mean values of estimated Ln probability of data (LnPD) for each *K* (A, C, E) and delta *K* (B, D, F) without prior population information. (A–B), results for the entire data set. (C–D), graphs for hierarchical analysis of the red cluster. (E–F), corresponding graphs for the green cluster.

Supplementary Table S1. Estimates of evolutionary divergence over cyt *b* sequence pairs between sample sites (*p*-distances).

	QU	WK	EK	KZ	KH	BA	RU	WG	ST	AN	SW	PW
QU	0											
WK	0.001	0										
EK	0.001	0.002	0									
KZ	0.000	0.001	0.001	0								
KH	0.003	0.002	0.004	0.003	0							
BA	0.001	0.001	0.003	0.001	0.002	0						
RU	0.001	0.001	0.002	0.001	0.003	0.002	0					
WG	0.001	0.001	0.002	0.001	0.002	0.001	0.001	0				
ST	0.001	0.001	0.002	0.001	0.002	0.000	0.001	0.001	0			
AN	0.001	0.001	0.002	0.001	0.003	0.001	0.002	0.001	0.001	0		
SW	0.001	0.001	0.002	0.001	0.003	0.001	0.002	0.001	0.001	0.001	0	
PW	0.001	0.001	0.002	0.001	0.002	0.001	0.001	0.001	0.000	0.001	0.001	0

Supplementary Table S2. Geographic distances (km) among sample sites.

	QU	WK	EK	KZ	KO	KH	BH	BA	RU	WG	ST	AN	SW	PW
QU	0	24.89	59.36	118.51	121.07	138.45	138.18	59.60	145.74	186.98	194.94	225.27	214.32	244.29
WK	24.89	0	55.80	121.60	124.18	140.04	140.47	77.42	165.05	189.68	206.53	236.57	221.51	254.05
EK	59.36	55.80	0	66.97	69.52	84.52	85.18	57.94	135.51	134.27	158.58	187.71	168.42	203.12
KZ	118.51	121.60	66.97	0	2.59	20.80	19.80	79.20	107.70	68.55	98.15	125.14	101.84	138.06
KO	121.07	124.18	69.52	2.59	0	18.53	17.33	81.32	107.77	65.97	96.27	123.05	99.39	135.75
KH	138.45	140.04	84.52	20.80	18.53	0	3.94	99.81	118.84	49.76	93.08	117.07	88.33	126.83
BH	138.18	140.47	85.18	19.80	17.33	3.94	0	98.08	115.08	49.22	89.62	114.01	86.17	124.28
BA	59.60	77.42	57.94	79.20	81.32	99.81	98.08	0	87.65	142.67	137.64	167.94	161.85	188.28
RU	145.74	165.05	135.51	107.70	107.77	118.84	115.08	87.65	0	134.01	84.08	108.84	124.69	133.14
WG	186.98	189.68	134.27	68.55	65.97	49.76	49.22	142.67	134.01	0	73.62	86.47	47.60	88.04
ST	194.94	206.53	158.58	98.15	96.27	93.08	89.62	137.64	84.08	73.62	0	30.35	43.49	51.77
AN	225.27	236.57	187.71	125.14	123.05	117.07	114.01	167.94	108.84	86.47	30.35	0	42.06	24.53
SW	214.32	221.51	168.42	101.84	99.39	88.33	86.17	161.85	124.69	47.60	43.49	42.06	0	40.48
PW	244.29	254.05	203.12	138.06	135.75	126.83	124.28	188.28	133.14	88.04	51.77	24.53	40.48	0

Supplementary Table S3. GenBank accession numbers for *Hyla arborea* CytB haplotypes Hy 1 to Hy 11.

Sequence ID	GenBank accession number
BankIt2634361 Seq1	OP690610
BankIt2634361 Seq2	OP690611
BankIt2634361 Seq3	OP690612
BankIt2634361 Seq4	OP690613
BankIt2634361 Seq5	OP690614
BankIt2634361 Seq6	OP690615
BankIt2634361 Seq7	OP690616
BankIt2634361 Seq8	OP690617
BankIt2634361 Seq9	OP690618
BankIt2634361 Seq10	OP690619
BankIt2634361 Seq11	OP690620